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Molecular phylogenetic analysis of five subfamilies of the Acrididae（Orthoptera：Acridoidea）based on the mitochondrial cytochrome b and cytochrome c oxidase subunit I gene sequences

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Abstract：The homologus sequences of the mitochondrial cytochrome b（*Cytb*）and cytochrome c oxidase subunit I（*COI*）genes were sequenced in 17 species representing five subfamilies（belonging to family Acrididae）：Melanoplinae，Catantopinae，Cyrtacanthacridinae，Oedipodinae and Gomphocerinae from China. The concatenated sequence from both genes was 1 998 bp in length，consisting of 1 266 bp and 732 bp for *COI* and *Cytb* respectively. In the concatenated sequence，A + T content was about 72.13%，and C + G only 27.87%. There are 889 sites conserved，1 109 sites were variable，and 838 sites were parsimony-informative in the variable sites. Phylogenetic trees were reconstructed with maximum parsimony，neighbor-joining，and maximum likelihood using *Erianthus versicolor* and *Erianthus* sp. of the Eumastacoidea as outgroups. Our results showed that the monophyly of the subfamilies Gomphocerinae and Catantopinae were not supported here. The phylogenetic trees did not entirely agree with the international taxonomic system of grasshoppers. The subfamily Oedipodinae clustered as one clade，which was supported as a monophyletic group. Based on the close relationships among four subfamilies，Gomphocerinae，Catantopinae，Cyrtacanthacridinae and Melanoplinae，we suggest that the four subfamilies should be considered to merge into one subfamily. Simultaneously，we found it was not very dependable to infer the phylogenetic relationships among subfamilies within the Acrididae based on the concatenated sequence from *Cytb* and *COI* genes.

Key words：Orthoptera；Acrididae；mitochondrial DNA；*Cytb* gene；*COI* gene；phylogeny

1 INTRODUCTION

The grasshoppers is a large group in the Orthoptera，comprising over 2 364 genera and 10 590 species worldwide（Flook and Rowell，1997）. Although phylogenetic relationship of this superfamily has been investigated from a variety of perspectives（Liu，1991；Xi and Zheng，1997；Xu and Zheng，1999a）and at different taxonomic levels，it is still unclear and becomes a hotspot of phylogenetic researches lately. Historically，the Acridoidea was divided into a number of families（Chopard，1951；Dirsh，1956；Bei-Bienko and Mishchenko，1963）with morphological characters. The fundamentals of these characters included：antennae（filiform，clavate and ensiform），tegmen，sound-producing organ，and tympanal organ. Yin（1982）established his own grasshopper taxonomy in China with these data，and divided Acridoidea into six families. In Xia's system（Xia，1998），Acridoidea was divided into eight families. But Sharov（1971）thought that it's not necessary to divide this family into so much and some characters should not been considered as family's diagnosis characters. From this，it can be seen that Chinese grasshopper taxonomic system is very different from the international system. Despite these apparent synapomorphies，the taxonomic status of this superfamily is still questioned.

Molecular techniques could offer useful informations to phyligenetic analysis. MtDNA sequence data have been proven useful for researching phylogenetic relationships of insects（Simon *et al*.，1994；Litzenberger and Chapco，2001，2003；Ren *et al*.，2002）. The cytochrome b（*Cyt b*），cytochrome c oxidase subunit I（*COI*），*COII*，16*S rDNA* and other markers based on mtDNA sequences were widely used in phylogenetic reseaches. Fragments of two mitochondrial genes，*Cytb* and *COI* have been used as phylogenetic markers in insects，*e.g.*，the nucleotide sequences of 685 bp from *Cytb* and 596 bp from *COI* have been determined for 36 Chironomus species from the Palearctic，or Holarctic，and Australasia. The concatenated sequence of 1 281 bp from both genes was

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used to investigate the phylogenetic relationships among these species（Guryev *et al*.，2001）. The concatenated（1 241 bp）sequences from both *Cytb* and *COI* genes were used to infer the phylogenetic relationships among seven *Sergentia* species（Chironomidae，Diptera）（Papoucheva *et al*.，2003）. Coeur d'acier *et al*.（2007）used partial sequences of *COI*/*COII* and *Cytb* genes to reconstruct the first phylogeny of *Aphis* and discuss the present systematics. For the grasshoppers，Flook and Rowell（1997）established a molecular taxonomic system of the Caelifera using 12*S* and 16S *rDNA* as markers. Litzenberger and Chapco（2001，2003）combined *Cytb*，*COI*，*COII* and *ND*2 to research the phylogenetic relationships within the tribal and subtribal groups in subfamily Melanoplinae. Liu and Jiang（2005）tested the phylogenetic relationships within Acridoidea based on 18*S rDNA*，and suggested that Acridoidea should be divided into four families: Pyrgomorphidae，Pamphagidae，Pneumoroidae and Acrididae. Huo *et al*.（2007）used *Cyt b* sequences to reconstruct the phylogenetic relationship of the family Acrypteridae，and their results supported the conclusions from 18*S rDNA* analysis（Yin *et al*.，2004; Liu and Jiang，2005），merging Catantopidae，Arcypteridae，Oedipodidae，Gomphoceridae，and Acrididae into one family Acrididae. Apparently，according to Chinese taxonomic system of Acridoidea，previous researches of Chinese scholars focused on elucidating relationships within the Acridoidea（Liu and Jiang，2005）and the family（Liu *et al*.，2005; Huo *et al*.，2007; Sun *et al*.，2006）and among subfamilies（Liu and Jiang，2005）. With respect to phylogenetic relationships among subfamilies，there are very little information in the literatures.

So we would like to use the concatenated sequence from *Cytb* and *COI* genes to infer the phylogenetic relationships among subfamilies within the Acrididae. Herein，according to the international system of Acridoidea，we present a study of the phylogenetic relationships and the monophyly of the five subfamilies from China，*i*. *e*.，Melanoplinae，Catantopinae，Cyrtacanthacridinae，Oedipodinae and Gomphocerinae. Our goals are to infer their phylogenetic relationships of some subfamilies within the family Acrididae，and to examine the validity of the concatenated sequence as marker in Acididae.

2 MATERIALS AND METHODS

2.1 Sample collection

A total of 17 recognized species of Acrididae was examined（Table 1）. These specimens were collected in the northwest and southwest of China. The classification was based on the web，Orthoptera Species File Online（http://osf2.orthoptera.org/HomePage.aspx）（Eades and Otte，2008）. The adult specimens of grasshoppers were frozen or presented in 100% ethanol.

Table 1 Collection locality and GenBank accession no. for species sequenced in this study

Species	Locality	GenBank accession no.	
		COI	*Cytb*
Melanoplinae			
Sinopodisma wuyishana	Qianshan，Jiangxi	EU366105	EU366106
Catantopinae			
Xenocatantops humilis	Chongzuo，Guangxi	EU366111	EU366112
Xenocatantops brachycerus	Qixia Mountain，Jiangsu	EU366109	EU366110
Catantops pinguis pinguis	Xiamen，Fujian	EU366081	EU366082
Apalacris varicornis	Qianshan，Jiangxi	EU366077	EU366078
Cyrtacanthacridinae			
Patanga apicerca	Laibin，Guangxi	EU366099	EU366100
Oedipodinae			
Bryodema luctuosum indum	Qingtongxia，Ningxia	EU366079	EU366080
Locusta migratoria manilensis	Xiamen，Fujian	EU366091	EU366092
Pternoscirta caliginosa	Chongzuo，Guangxi	EU366103	EU366104
Oedaleus infernalis	Changbai Mountain，Jilin	EU366095	EU366096
Trilophidia annulata	Wuyishan，Fujian	EU366107	EU366108
Aiolopus thalassinus tamulus	Nanjing，Jiangsu	EU366075	EU366076
Gomphocerinae			
Omocestus haemorrhoidalis	Diebu，Gansu	EU366097	EU366098
Stenobothrus carbonarius	Diebu，Gansu	EU366093	EU366094
Pezohippus biplatus	Wenxian，Gansu	EU366101	EU366102
Aeropus sibiricus	Nanshan，Xinjiang	EU366085	EU366090
Chorthippus sp.（similar to *Chorthippus chinensis*）	Diebu，Gansu	EU366083	EU366084
Erianthinae			
Erianthus versicolor	Fangcheng，Guangxi	EU366086	EU366088
Erianthus sp.（similar to *Erianthus versicolor*）	Fangcheng，Guangxi	EU366087	EU366089

2.2 DNA extraction

Total genomic DNA was extracted from hind femora of adult specimens preserved or frozen using a simple proteinase K/SDS method. Before incubation, the samples were marinated in ddH_2O, water was changed 3 - 4 times a day. Scissored tissues were resuspended in 400 μL 0.01 mol/L Tris (pH 8.0), 0.1 mol/L EDTA (pH 8.0), 0.05 mol/L NaCl, 0.1% SDS, 5 μL proteinase K and incubated at 50℃ for 8 - 10 h. The digested samples were phenol-extracted, ethnol-precipitated once more, and redissolved in 30 μL ddH_2O (pH 8.0). DNA quality was checked on a 1% - 1.5% agarose/tris-borate-EDTA gel. All DNA samples were stored at -20℃.

2.3 PCR amplification and sequencing

The fragments of *COI* and *Cytb* were amplified from the 17 individuals using primers (Table 2) from Simon *et al*. (1994).

Table 2 The primers used in this study

Gene	Primer name	Sequence (5′ - 3′)	Product length (bp)
COI	UEA3	TATAGCATTCCCACGAATAAATAA	340
	UEA4	AATTTCGGTCAGTTAATAATATAG	
	UEA5	AGTTTTAGCAGGAGCAATTACTAT	680
	UEA8	AAAAATGTTGAGGGAAAAATGTTA	
	UEA7	TACAGTTGGAATAGACGTTGATAC	700
	UEA10	TCCAATGCACTAATCTGCCATATTA	
Cyt b	CB1	TACGTTTTACCATGAGGTCAAATATC	750
	TS1	ACTTCTTTTCTTATGTTTTCAAAAC	

PCR reactions were carried out in 30 μL volume containing 10 × reaction buffer 3 μL, 25 mmol/L $MgCl_2$ 2 μL, 2 mmol/L dNTPs 2 μL, primers 10 μmol/L 1 μL, ddH_2O 19.8 μL, 1 U taq DNA polymerase, template 1 μL (containing DNA 20 - 50 ng). Amplification was performed under the following conditions: an initial denaturation step at 95℃ for 5 min; 30 cycles of 30 s 95℃, 30 s 48℃, 30 s 72℃; and a final extension step at 72℃ for 7 min. Amplification products were examined on 1.0% agarose gels and purified using PCR clean-up kit or DNA gel extraction kit according to conditions and then sequenced using Dye-labeled terminator on an ABI377 and ABI3730 automated sequencer.

2.4 Data analyses

Sequences were collected manually and the ambiguous parts were deleted. The collected sequences were transferred into amino acids and aligned using software package MEGA 3.0, in order to assess potential saturation for each separate codon position of the *COI* and *Cytb* sequences among all taxa.

The checked sequences of the two genes joined together, gaps of the head and the end were treated as missing. The variability and heredity distances of the combined sequence were based on Kimura-2-parameter model, and then were calculated using MEGA 3.0 (Kumar *et al*., 2001). The numbers of variable and parsimony-informative sites were also calculated using MEGA 3.0 Software DAMBE (Xia and Xie, 2001) was used to calculate the saturation of sequence variations.

2.5 Phylogenetic trees

Three different methods of phylogenetic analyses, maximum likelihood (ML), neighborjoining method (NJ) and maximum parsimony (MP) analysis were performed using PAUP* 4.0b10 (Swofford, 2002). For phylogenetic analysis, both *Erianthus versicolor* and *Erianthus* sp. of the Eumastacoidea were used as outgroups. Data were initially analyzed with neighbor-joining (NJ) analyses. Maximum-parsimony (MP) (Fitch, 1971) analyses were performed under the heuristic search option with 1 000 random-addition replicates and TBR branch swapping. Each nucleotide was treated as an unordered character with four alternative states, and gaps were considered as missing data in all analyses (Allard and Carpenter, 1996; Cibois *et al*., 1999). The maximum-likelihood (ML) (Felsestein, 1981), parameters were estimated using hierarchical likelihood-ratio tests as implemented in Modeltest 3.06 (Posada and Crrandall, 1998). The best model chosen was GTR + G, which was showed in Table 3.

Table 3 Nucleotide substitution model parameter estimates for hierarchical likelihood ratio test (hL RT)

- LnL	π (N)	G	RAC	RAG	RAT	RCG	RCT	RGT
14464.3008	Equal	0.2911	2.9851	9.3401	7.2276	2.8685	33.4568	1.0000

3 RESULTS

3.1 DNA sequence compositions

The combined sequence data was 1 998 bp, consisting of 1 266 bp and 732 bp for *COI* and *Cytb* respectively, with 1 109 variable sites and 889 conserved sites; among the variable sites, 838 were parsimony-informative sites. The R (transition/transversion) was 1.064. The average contents of A, T, G, C were 35.09%, 37.04%, 11.50%, 16.37%, respectively. A + T content was about 72.13%, and G + C only 27.87%, these are consistent in general to be highly A + T biased in insects (Crozier and Croizer, 1993; Flook *et al*., 1995; Ding and Huang, 2008; Liu and Huang, 2008).

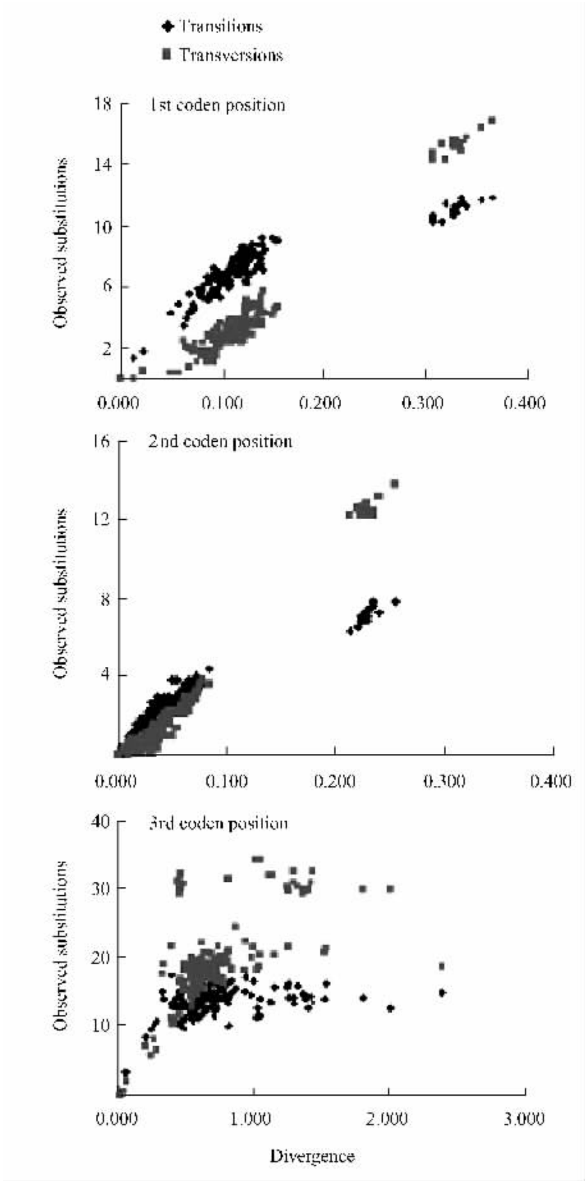

Fig. 1 Patterns of substitutions with *Cytb* and *COI*
Transitions or transversions among pairwise comparisons of sequences from 17 species of Acrididae and two outgroups plotted against divergence，performed by DAMBE.

The observed substitutions were plotted against the divergence（Fig. 1）. Among the 17 species，the 1st and 2nd codon positions all showed that the transversion was still straightforward，but the transitions reached a platform，which indicated the transversion did not reach saturation but transitions did so. Whereas，for the 3rd position，the transitions and transversion all reached a platform，which indicated that both reached saturation. “Noisy” third positions were eliminated as they would influence the results of following analysis like MP analysis.

The maximum and minimum *p*-distance of slected species within Acrididae caculated by using MEGA 3.0 was 0.219（between *Locusta migratoria manilensis* and *Apalacris varicornis*）and 0.017（between *Xenocatantops humilis* and *Xenocatantops brachycerus*）respectively. The maximum p-distance was between the genetic distances within Oedipodinae and the genetic distances within Catantopinae，consistent with the topological structures of the MP，ML and NJ trees.

3.2 Molecular phylogenetic trees

The topologies of the NJ and MP trees（Figs. 2 and 3）were slightly similar，different in the bootstrap values of some clades and positions of some species. The two trees all consist of two clades，among them Apalacris varicornis of Catantopinae formed one clade，other species formed another large clade. In the large clade，all species of Oedipodinae formed one clade and the other species formed another clade，which consist of the species of Gomphocerinae，Melanoplinae and Crytacanthacridinae，and the species of Catantopinae expect *A*. *varicornis*（Figs. 2 and 3），the species of Gomphocerinae except *Omocestus haemorrhoidalis* clustered together，the species of Catantopinae except *A*. *varicornis* also clustered together，and their bootstrap values were very high（≥99%）；but the positions of *Patanga apicerca*（Cyrtacanthacridinae），*Sinopodisma wuyishana*（Melanoplinae）and *O*. *haemorrhoidalis* of Gomphocerinae are unstable. The consistency index（*CI*）and the retention index（*RI*）for the MP tree was 0.5204 and 0.5100，respectively.

ML tree（Fig. 4）was different from the other trees in the topological structure. As shown in Fig. 2，ingroups formed two large clades，among them the Oedipodinae species formed the first large clade and the other species formed the second large clade. In the second large clade，the species of Catantopinae formed a small clade with high values（96%）except *A*. *varicornis*；the species of Gomphocerinae except *Omocestus haemorrhoidalis* formed another small clade with *Patanga apicerca* of Crytacanthacridinae.

4 DISCUSSION

4.1 The systematic status of the Oedipodinae

The subfamily Oedipodinae was always resolved as a monophyly. In this study，all analysis supported it as a monophyly，although the bootstrap values were lower（Figs. 2 – 4）. This conclusion is consistent with grasshopper morphology classification of Chinese and international taxonomic systems. Liu and Jiang（2005）also got the same result using 18*S rDNA*. These results indicated that the monophyly of Oedipodinae was affirmative and stable.

Although we supported this subfamily as a

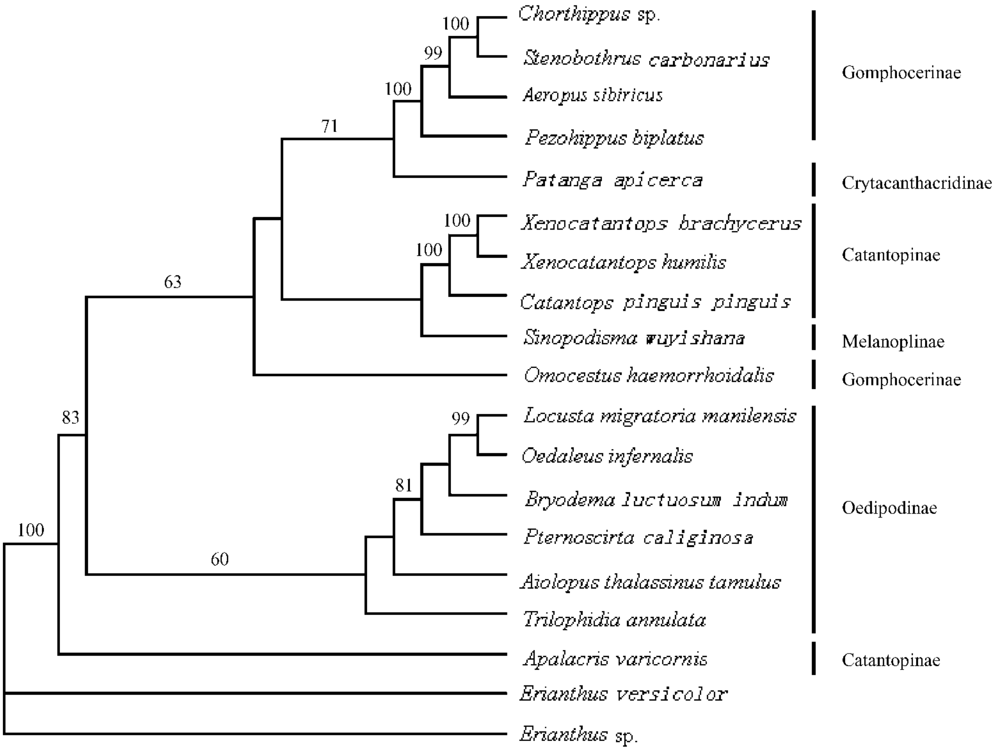

Fig. 2　NJ tree resulting from analysis of the joined *COI* and *Cytb* sequences data of 17 grasshoppers

The numbers above the branches noted the bootstrap values of 1 000 replicates which were more than 50.

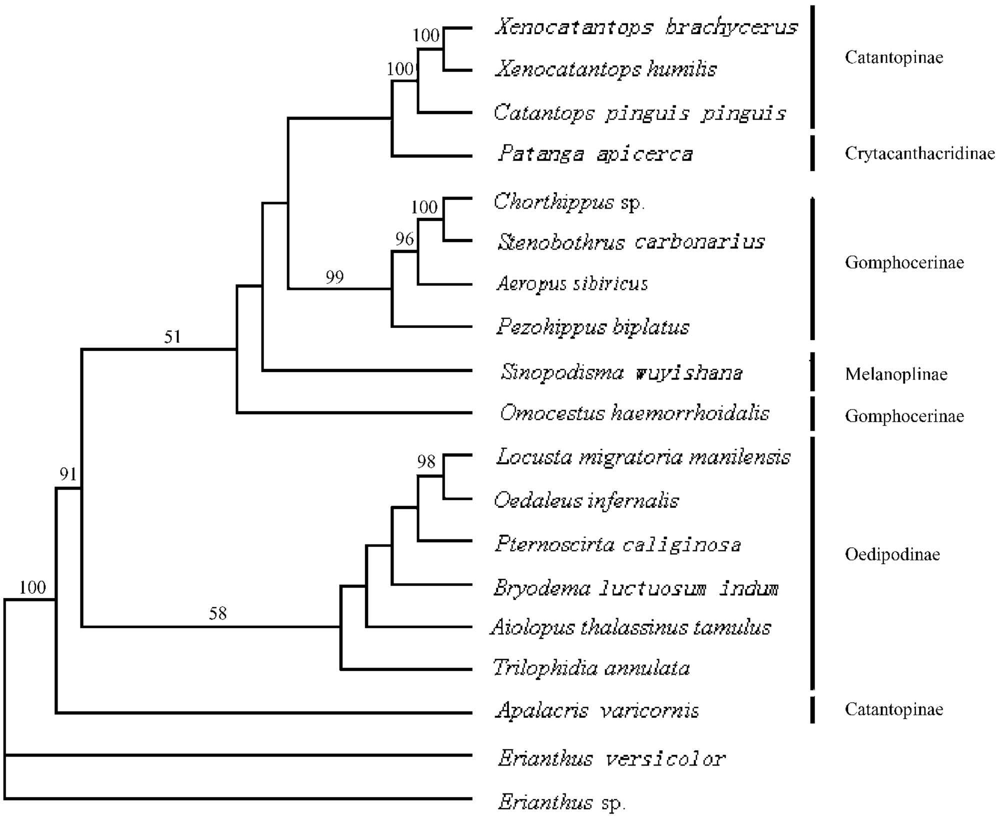

Fig. 3　MP tree resulting from analysis of the joined *COI* and *Cytb* sequences data

The numbers above the branches noted the bootstrap values for 1 000 replicates which were more than 50.

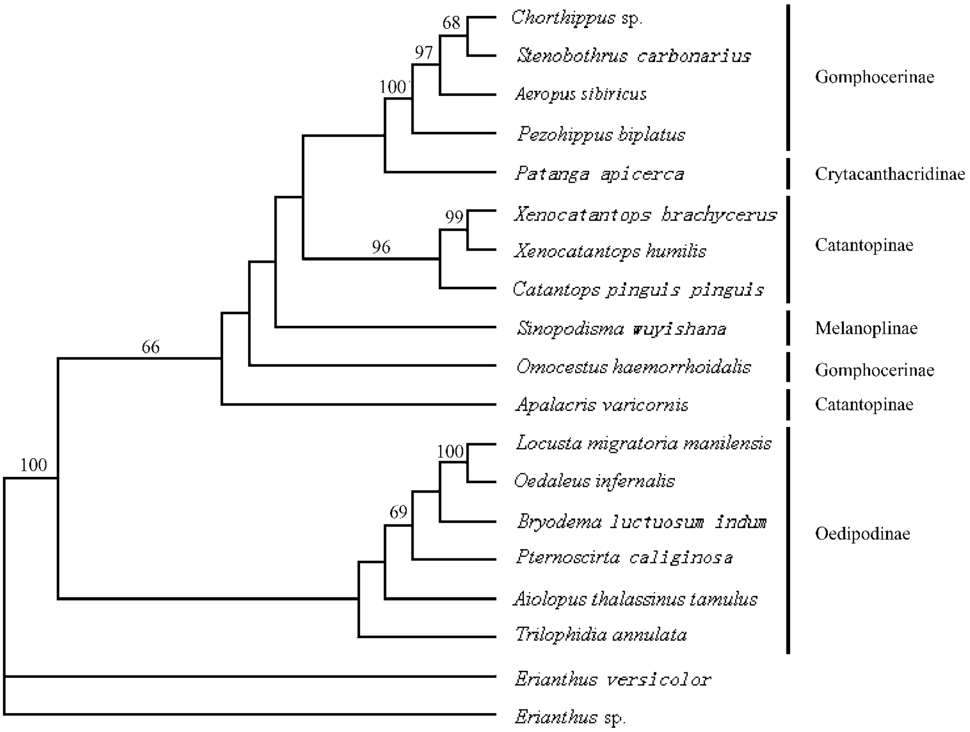

Fig. 4 ML tree resulting from analysis based on the joined *COI* and *Cytb* gene data
The numbers above the branches noted the percentages bootstrap values of 100 replicates which were more than 50.

monophyletic group, we can not confirm the phylogenetic trend. As shown by all the topological structure, the branch order of Acrididae, which Oedipodinae species formed, was in the bottom and near to the outgoups. Compared with the other subfamilies, we suggested that this subfamily was not so developed as the Gomphocerinae and Cyrtacanthacridinae. Ren *et al*. (2002) and Yin *et al*. (2003) had got the same result that Oedipodinae was relatively independent subfamily in Acrididae, especially when using *Cytb* as a marker. Ren *et al*. (2002) found this subfamily was the second seminal subfamily. On the basis of the female subgenital plate and sperm, Xu and Zheng (1999a, 1999b) and Xi and Zheng (1997), respectively, proposed that the Oedipodinae, Gomphocerinae, Arcypterinae and Catantopinae were more developed subfamilies. Here we agree with that Gomphocerinae is more developed.

4.2 The relationships of the other four subfamilies

From the toplogies shown in the three trees, it appeared that Catantopinae and Gomphocerinae constituted one paraphyletic group. The two species, *A*. *varicornis* (Catantopinae) and *O*. *haemorrhoidalis* (Gomphocerinae), always out of the main clade constructed by their subfamilies. In the three trees, *A*. *varicornis* was far away from the main clades. The bootstrap values for *A*. *varicornis* were high in NJ (83%), MP (91%) and low in ML analysis (50% lower). It seemed that this species was relatively independent, not belonging to Catantopinae, and could be merged into another subfamily. So our results didn't support that Catantopinae and Gomphocerinae were monophyletic groups. In a previous study, based on the 16S rDNA sequences, Liu *et al*. (2005) found that monophyly of Catantopidae from China was not supported by phylogenetic trees. Based on COII gene sequence, Ma and Huang (2006) concluded that the relationships among Calliptaminae, Cyrtacanthacridinae, Eyprepocnemisinae and Catantopinae were close and the topologies showed that these subfamilies combined as a large clade. Thus the monophyly of subfamilies Catantopinae and Gomphocerinae should be supported by more evidences.

The two subfamilies, Melanoplinae and Cyrtacanthacridinae, each including only one species, their monophyly should be confirmed by more species. According to the results of grasshopper chromosomal study, Ma *et al*. (2000) thought that the Catantopidae and Oedipodidae can be grouped together. In the same way, it is obvious that the Gomphocerinae, Cyrtacanthacridinae, Catantopinae and Melanoplinae had very close relationships between each other from our results (Figs. 2 – 4). The species within one subfamily were grouped first and there were very high bootstrap values on the main clades，although two species were out of them. In these four subfamilies，Gomphocerinae seemed to be the most evolved，Cyrtacanthacridinae was its sister group. Based on the follicle morphology analysis，Ma *et al*.（2001）concluded that the Catantopidae was a relatively evolved taxon. From Fig. 3，our results on evolutionary position of Catantopihae was similar to Ma's idea（2001）. Therefore，the relationships of these four subfamilies can not be confirmed by our research. So we suggest that the four subfamilies should be combined into one subfamily.

In summary，from our results and above analysis，we do not agree with the other Chinese scholars in dividing the Acridoidea into eight families，but agree with the international taxonomic system at family level. At subfamily level，however，we do not support either the international system or the Chinese system of grasshoppers. Indeed，if we want to know definitely how many subfamilies should be divided within the Acrididae，we need to do more works in future.

4.3 Validity of the concatenated sequence from *Cytb* and *COI* genes used to infer the phylogenetic relationships among subfamilies within the Acrididae

It is well-known that the long sequence includes more phylogenetic information than that of the short sequence. So the concatenated sequence is popularly used to study the systematics in different taxonomic bank today. In insects，some scholars have obtained acceptable results based on the concatenated sequence from *Cytb* and *COI* genes（Guryev *et al*.，2001；Papoucheva *et al*.，2003；Coeur d'acier *et al*.，2007）. For example，monophyly of the genus *Chironomus* is supported by a bootstrap value of 100% at the basal branch. Six clusters of species have been revealed with high bootstrap values supporting both monophyly of each cluster and the validity of the branching order within each cluster（Guryev *et al*.，2001）. Monophyly of both *Sergentia* and the Baikalian endemic species was well supported（Papoucheva *et al*.，2003）. In Coeur d'acier *et al*.（2007），the monophyly of the subgenus *Bursaphis* and of the tree major species groups，black aphid，black backed aphid and frangulae-like species was recovered by all phylogenetic analyses. However，their data suggested that the nominal subgenus was not monophyletic. In the three examples，the concatenated sequence from *Cytb* and *COI* genes were all used to infer the relationships among species within one genus. In this study，monophyly of the subfamily Oedipodinae was well supported，but not for the other four subfamilies. Therefore，we speculate that the evolutionary rate of the *Cytb* and *COI* genes are fast，are suitable to study the relationships among or within genus，and not useful to infer the phylogenetic relationships of grasshoppers among subfamilies and above.

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基于线粒体基因 *Cytb* 和 *COI* 的蝗科中五亚科分子系统发育关系分析

（直翅目：蝗总科）

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摘要：本研究基于 *Cytb* 基因和 *COI* 基因的部分序列来推断 17 种蝗虫之间的系统发育关系。这 17 种蝗虫均采自国内，代表了蝗科（Acrididae）5 个亚科：黑蝗亚科（Melanoplinae）、斑腿蝗亚科（Catantopinae）、刺胸蝗亚科（Cyrtacanthacridinae）、斑翅蝗亚科（Oedipodinae）和大足蝗亚科（Gomphocerinae）。采用联合序列方法进行分析，结果显示：*Cytb* 和 *COI* 联合序列长度为 1 998 bp，其中 A 和 T 总含量为 72.13%，G 和 C 总含量为 27.87%。联合序列共包含了 889 个保守位点，1 109 个变异位点，在这些变异位点中有 838 个简约信息位点。系统发生树采用邻接法（NJ）、最大简约法（MP）和最大似然法（ML）进行构建。使用蜢总科的变色乌蜢 *Erianthus versicolor* 和 *Erianthus* sp. 两个种作为外群。结果表明：大足蝗亚科和斑腿蝗亚科的单系性没有得到支持。斑翅蝗亚科内部各种聚成一个大支，在本研究中该亚科的单系性得到支持，与前人的研究结论相同。大足蝗亚科、斑腿蝗亚科、刺胸蝗亚科和黑蝗亚科这 4 科关系非常近，可以考虑将其合并为一个亚科。同时，我们发现基于 *Cytb* 和 *COI* 基因联合序列推断蝗科内各亚科间的系统发生关系并不十分可靠。

关键词：直翅目；蝗科；线粒体 DNA；*Cytb* 基因；*COI* 基因；系统发育

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